Differential expression of pathogen-responsive genes encoding two types of glycine-rich proteins in barley

Antonio Molina, Montaña Mena, Pilar Carbonero and Francisco García-Olmedo
Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología - UPM, E.T.S. Ingenieros Agrónomos, E-28040 Madrid, Spain

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Abstract

Gene-specific probes (3′ ends of cDNAs) were obtained from barley cDNAs encoding two types of glycine-rich proteins: HvGRP2, characterized by a cytokeratin-like and a cysteine-rich domain, and HvGRP3, whose main feature was an RNA-binding domain. Expression of genes *Hvgrp2* and *Hvgrp3*, which are present at one (or two) copies per haploid genome, was ubiquitous and gene *Hvgrp3* was under light/darkness modulation. Cold treatment increased *Hvgrp2* and *Hvgrp3* mRNA levels. Methyl jasmonate (10 μM) switched off the two genes. Expression of *Hvgrp2*, but not that of *Hvgrp3*, was induced by ethylene treatment (100 ppm). Fungal pathogens *Erysiphe graminis* and *Rhynchosporium secalis* increased the mRNAs levels of the two genes, both in compatible and in incompatible interactions, while bacterial pathogens did not.

Introduction

Glycine-rich proteins (GRPs) have been found in different tissues from many plant species [5, 7, 9–11, 13, 15, 17, 18, 20, 24, 26, 28, 33–36, 41, 46] and have been classified into different families [10, 21]. A distinct GRP type (CL-GRP family) is represented by HvGRP1 from barley, whose amino acid sequence, which has been deduced from a genomic clone [36], has a cytokeratin-like domain that consists of glycine stretches with interspersed tyrosine residues, followed by a cysteine-rich C-terminal domain, and preceded by a charged N-terminal domain and a putative signal peptide. A C-terminal cysteine-rich domain has also been found in certain GRPs from *Nicotiana tabacum* [46], *Arabidopsis thaliana* [34], and *Petunia hybrida* [26], but these lack the cytokeratin-like domain present in HvGRP1, although they have short glycine-rich domains. Other GRPs from *A. thaliana* [34], *P. hybrida* [9], and bean [20] have glycine-rich domains like that of HvGRP1, but lack the cysteine-rich domain. Subcellular location of HvGRP1 is unknown, but the presence of a signal peptide suggests a similar cell-wall location as some other GRPs [19, 20, 37, 40, 47].

A second GRP type, which is able to bind RNA (RNA-GRP family), has been more extensively studied and is characterized by a domain with two typical RNA-binding motifs [7, 10, 11, 15, 17, 18, 35, 41], which is also present in some non-GRP plant proteins [25, 29], as well as in some proteins from yeast, insects, and mammals with and without glycine-rich domains [32]. RNA-GRPs specifically bind poly(U) and poly(G) [27], have high affinity for the RNA of the same cells where they are present [18], and are located in the nucleolar compartment [1, 17].

Expression of genes encoding CL-GRPs has not been previously investigated, whereas genes encoding RNA-GRPs are known to be developmentally regulated in other plant species [15, 17]. Additionally, given genes of the latter type have been shown to be under circadian rhythm modulation [7, 17], to respond to different stresses, such as cold [7], drought [7, 17], and wounding [5, 35, 41], and to be induced by external treatments with abscisic acid [15], ethylene [35], and $HgCl_2$ [11].

In the course of our studies on the variability of barley defense responses, we have investigated the expression of two pathogen-responsive *grp* genes, *Hvgrp2* and *Hvgrp3*, encoding a CL-GRP and an RNA-GRP respectively.

Materials and methods

Cloning of cDNA

Developing barley endosperm from *Hordeum vulgare* L. cv. Abyssinian 2331, collected at 14 days after pollination (dap), was the source of the poly(A)$^+$ mRNA for the construction of the cDNA library in the lambda vector NM1149 [36]. The library was screened at 58 °C on nylon membranes (Hybond N; Amersham) with the oligonucleotide probe (5′-TA-(C/T)C(A/C)NGGNCA(C/T)GGNGGNG(A/G)N-3′) corresponding to the repetitive amino acid sequence Y(P/H)GHGG(E/G) of HvGRP1 [36]. The clones selected were subcloned into M13mp18-19 vectors and their cDNAs were sequenced by the dideoxy chain-termination method [38].

Southern and northern blot analyses

DNAs isolated from cv. Betzes as described [42] and digested with *Bam*HI, *Eco*RI and *Hin*dIII endonucleases, were subjected to electrophoresis in 0.8% agarose, and transferred onto Hybond N membranes (Amersham). Hybridizations were carried out at 65 °C in 0.5 M NaH_2PO_4, 1% SDS, 1 mM EDTA, 100 μg/ml salmon sperm DNA, as previously described [8].

Total RNA samples were purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M lithium chloride [23]. Electrophoresis was carried out on 7.5% formaldehyde/agarose gels, which were blotted onto Hybond N membranes (Amersham). Hybridization and washes were carried out at 65 °C according to Church and Gilbert [8]. Ethidium bromide (40 μg/ml) was included in the sample loading buffer to allow UV flight detection. Equal sample loads were checked by densitometry (INH Image Programme, BioRad) of northern blots obtained by hybridization with cDNA probe encoding 25S rRNA from barley. Positive signals in northern blot obtained with the two *Hvgrp* probes were quantified after two days exposure, by densitometry using the INH Image Programme (BioRad), or after overnight exposure using the Phosphor Analyst Programme (BioRad).

Sequence-specific 3′-end probes for both *Hvgrp* genes were obtained by subcloning the 3′-terminal *Xho*I-*Eco*RI fragments of *Hvgrp2* (280 bp; probe 2) and *Hvgrp3* (200 bp; probe 3) into pBluescript (Fig. 1A, B). A probe corresponding to a barley leaf thionin was the gift of Dr A. Segura (ETSIA, Madrid). The jasmonate induced protein (JIP60) probe was obtained by the polymerase chain reaction, using oligonucleotide primers designed from its EMBL database sequence. Hybridization probes were prepared using the Random Primer Labeling Kit (Boehringer) and αP^{32}-dATP (Amersham).

External treatments

Barley cv. Bomi plants used throughout these treatments were grown on vermiculite at 22 °C day/18 °C night with a 16 h light/8 h darkness regime. Low-temperature treatment was carried out with 13-day-old plants essentially as previously described [12]: shoot apex and leaves were collected 2 and 7 days after exposure to 4 °C. Water deprivation was achieved by leaving 7-day-old plants on the bench until the appearance of clear wilting symptoms (12 h). Seven-day old plants were floated on a 10 μM solution of methyl jasmonate (Bedoukian Research) as described [2], and leaf ant root samples were collected at 6 h, 15 h, 24 h and 48 h. Abscisic acid (0.1 mM; Sigma), sodium salicylate (40 mM; Sigma), and ethylene (100 ppm) treatments were carried out on 7-day-old plants as reported previously [30]. A solution of 2,6-dichloro-isonicotinic acid (15 mM; Ciba-Geigy, Basel, Switzerland) was formulated with a wettable powder carrier and sprayed on 7-day-old plants; the wettable powder alone was used for the mock treatment. Leaves were collected at 12 h, 30 h and 84 h after treatment.

Infection with pathogens

Inoculation of barley cv. Pallas (line PO3, carrying the resistance gene *Mla6*) with the virulent (CC143; *vir6*) and avirulent (CC142; *Av6*) isolates of *Erysiphe graminis* was carried out by Drs L. Boyd, P.H. Smith and K.M. Brown at the John Innes Centre (Norwich, UK). Mildew spores were blown and allowed to settle on the leaves as reported [4]. Experiments with *Rhynchosporium secalis* (isolate US238.1) were done on barley cvs. Atlas46 (carrying resistance gene *Rh3*) and Atlas (near isogenic line lacking the resistance

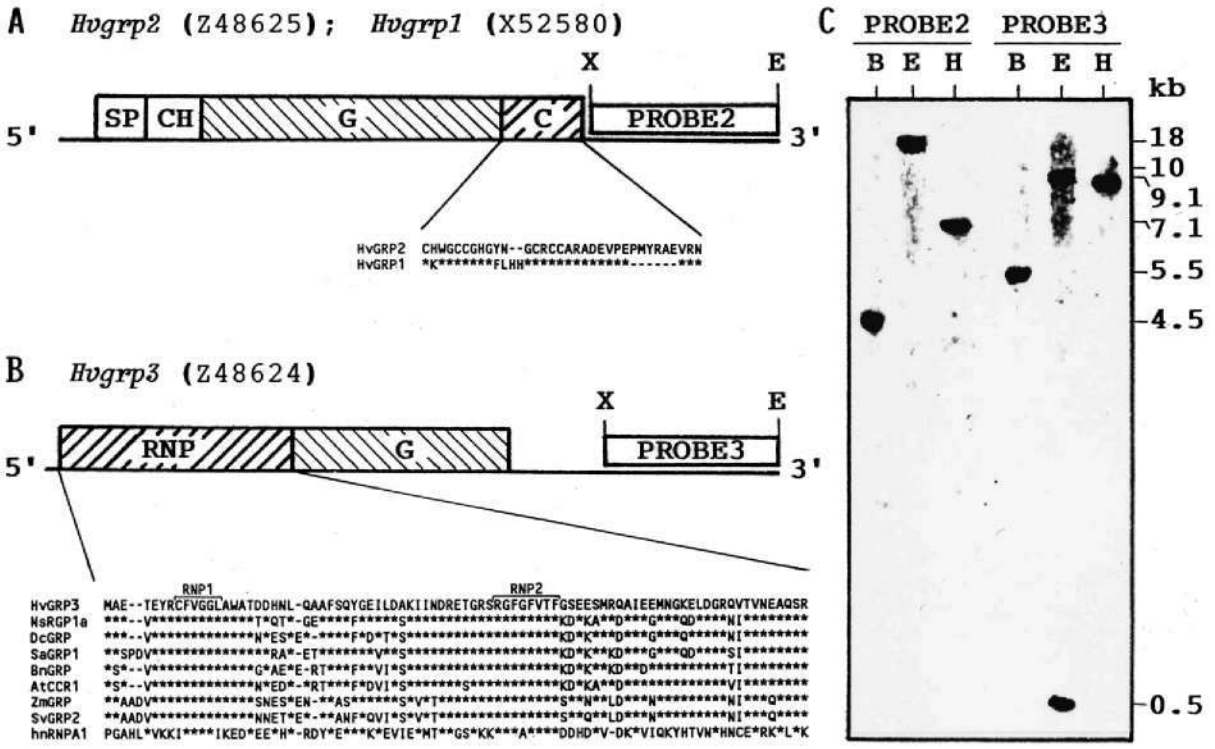

Figure 1. Two types of GRPs from barley. **A**. Schematic representation of CL-GRPs, based on genes *Hvgrp1* (EMBL accession number X52580; [32]) and *Hvgrp2* (Z48625). The signal peptide (SP), the charged N-terminal domain (CH), the glycine-rich domain (G), the cysteine-rich domain (C), and the region used as sequence-specific probe (Probe 2), are indicated. Identical residues in the alignment of the cysteine-rich domain of both proteins are marked by stars (*) and gaps by dashes (–). Restriction sites flanking the Probe 2 region were *Xho*I (X) and *Eco*RI (E). **B**. Representation of RNA-GRPs, based on the barley *Hvgrp3* sequence (Z48624). The RNA-binding domain (RNP), including the conserved RNP1 and RNP2 motifs, the glycine rich domain (G), and the region used as sequence-specific probe (Probe 3) are indicated. The RNP domain of HvGRP3 has been aligned with those present in NsGRP1a [18], DcGRP [41], SaGRP1 [17], BnGRP (Z14143), AtGRP7 [7], ZmGRP [15], and SvGRP2 [10] from plants, and that of human hnRNPA1 [6]. **C**. Southern blot analysis of *HvGrp2* and *HvGrp3* genes. DNA samples (15 μg each) from barley cv. Betzes were digested with the restriction endonucleases *Bam*HI (B), *Eco*RI (E) and *Hin*dIII (H). Size markers were fragments of the λ phage digested with restriction endonucleases *Eco*RI and *Hin*dIII.

gene) by Drs M. Hahn, S. Jüngling and W. Knogge at the Max-Planck Institut (Köln, Germany). Air inoculation was carried out as described [16].

Leaves of 7-day-old barley cv. Bomi plants, grown as for the external treatment, were infiltrated with the compatible bacteria *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae* pv. *japonica*, *Xanthomonas campestris* pv. *translucens* or *X. campestris* pv. *hordei*, or with the incompatible one *P. syringae* pv. 153 (10^7 bacteria per ml in 10 mM $MgCl_2$), or with 10 mM $MgCl_2$ alone. Leaf samples were taken up to 72 h after inoculation and immediately frozen in liquid nitrogen.

Results

Characterization of cDNA clones

Two cloned cDNAs (Z48625, Z48624), which were isolated from a barley developing endosperm library, using an oligonucleotide probe encoding the glycine repeat of HvGRP1 [32], were sequenced and found to respectively encode a CL- and an RNA-GRP, whose distinctive features are highlighted in Fig. 1A, B. The deduced amino acid sequence of HvGRP2 (Z48625) was highly similar (85% identity; 91% similarity) to that of previously reported HvGRP1 [32], although the untranslated 3′ ends were quite divergent (less than 30% coincident nucleotides). The deduced sequence for HvGRP3 (Z48624) was clearly homologous to RNA-GRPS previously described in other plant spe-

cies. Specific probes were derived from the divergent 3′ regions of the two cloned cDNAs and used for Southern analysis and to investigate expression of the corresponding genes. Southern blot hybridization patterns were consistent with the presence of one (or two) copies of each of these genes per haploid genome (Fig. 1C).

Expression of genes HvGrp2 *and* HvGrp3

Expression of the two genes, evaluated by northern blot, occurred in all plant tissues and organs investigated (Fig. 2A). Gene *Hvgrp3* (probe 3) was under light/darkness modulation, while *Hvgrp2* (probe 2) was not (Fig. 2B). The effect of light on gene *Hvgrp3* was confirmed by the fact that, after 8 h of exposure to light, initial mRNA levels were recovered within the first 2 h of darkness (not shown), which indicated that the variation of mRNA levels was not strictly circadian but did not exclude a circadian oscillation superimposed on the light effect. Additional differences were that *Hvgrp2* mRNA levels decreased with age, while those of *Hvgrp3* were less variable throughout, and that *Hvgrp2* expression levels in endosperm were relatively low while those of *Hvgrp3* mRNA were high (Fig. 2A).

Effects of physical treatments on the expression of both genes were studied in cv. Bomi. Cold temperatures significantly increased the two types of GRP mRNAs in leaves and in the shoot apex (Fig. 3). Under these conditions, a sucrose synthase gene (*Ss1*) was markedly induced and genes encoding lipid transfer proteins were not (data not shown). In contrast, expression of genes *Hvgrp2* and *Hvgrp3* was not altered by either drought or wounding (data not shown). Thionin genes, used as positive controls, were greatly induced by drought, and a gene encoding a ribosome inactivating protein (JIP60) was rapidly induced by wounding (data not shown).

Methyl jasmonate drastically decreased steady-state mRNA levels of both *grp* genes in leaves and roots (Fig. 4). Under the same conditions, genes encoding lipid transfer proteins were similarly affected and thionin genes were markedly induced (not shown). Expression of gene *Hvgrp2* was increased by ethylene, while that of gene *Hvgrp3* was not affected (Fig. 5). Treatments with abscisic acid (0.1 mM), salicylate (40 mM) or 2,6-dichloro-isonicotinic acid (40 mM), had no significant effects on the expression of either gene (data not shown). Expression of gene *HvPr1* [16], used as positive control was significantly elicited in response to the last three treatments.

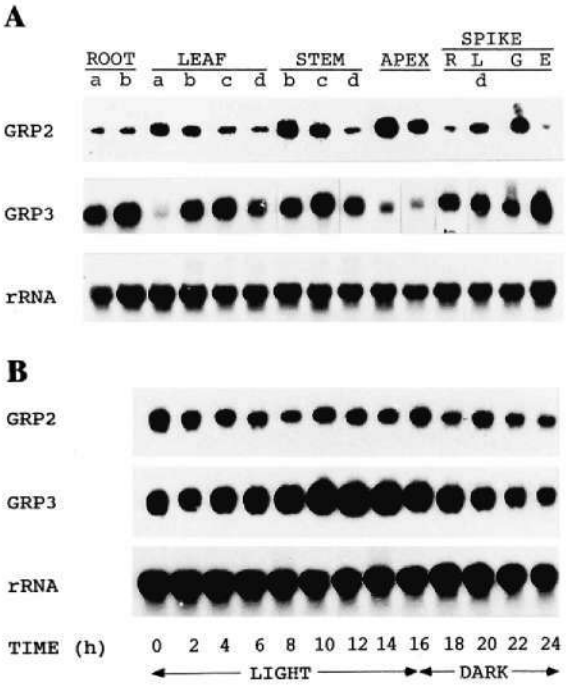

Figure 2. Expression of genes *Hvgrp2* and *Hvgrp3* in barley cv. Bomi. Northern blot analysis of total RNAs (7.5 μg per lane). **A**. Samples were collected from the indicated parts of the plant at the following times after the start of germination: a (4 d), b (9 d), c (20 d), and d (90 d). Spikes were separated into rachis (R), lemma plus palea (L), grain coats (G) and endosperm plus embryo (E). Blots correspond to one of three experiments with similar results. Hybridization with a 25S rRNA probe was used as a control of sample loading. **B**. Effect of light on the expression of *Hvgrp2* and *Hvgrp3* genes. Leaves of 7-day-old plants grown on 16 h ligth/8 h darkness were taken at the times indicated. Blots correspond to one of three experiments with similar results. Hybridization with a 25S rRNA probe was used as a control of sample loading.

Induction by pathogens

The effect of infection with fungal and bacterial pathogens on expression of the two *grp* genes was investigated in both compatible and incompatible interactions. Both genes responded to the fungal pathogens *E. graminis* and *R. secalis* (Fig. 6). Though consistent, the response to infection of gene *Hvgrp3* was only minor and was superimposed on the light/darkness modulation (Fig. 6A). Expression increases observed in the fungal compatible interactions did not differ significantly from those in the incompatible ones. No induction of either *grp* gene was observed upon infection with the compatible and incompatible bacterial strains tested (not shown), which had been chosen because of the diversity of their effects on expression of

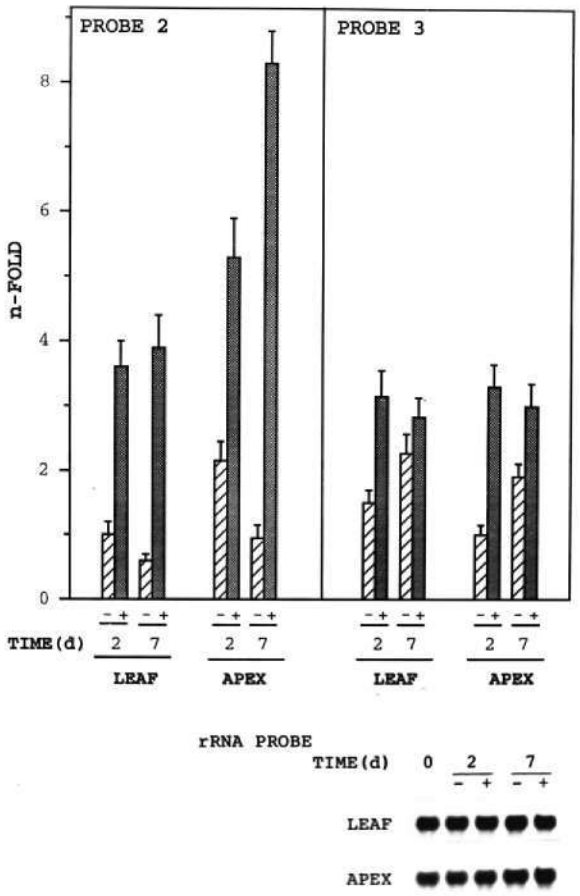

Figure 3. Responses of genes *Hvgrp2* and *Hvgrp3* to cold treatment. Thirteen-day-old plants were placed at 25 °C (−) or 4 °C (+) for the times indicated. Northern blot filters were quantified after overnight exposure using the Phosphor Analyst Programme. Signals obtained with each probe for 13-day-old leaves were given the arbitrary value of 1. Values represent the mean of three independent experiments, and bars indicate the standard errors of the mean. Hybridization with a 25S rRNA probe was used as a control of sample loading.

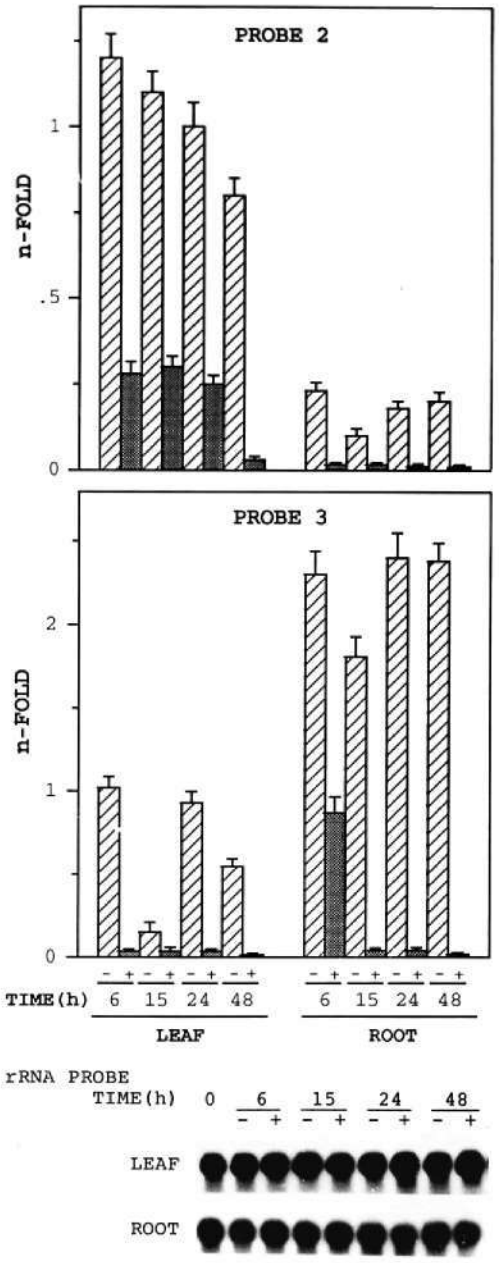

Figure 4. Effect of methyl jasmonate on the expression of genes *Hvgrp2* and *Hvgrp3*. Seven-day-old plants were floated on water (−) or in a 10 μM solution of methyl jasmonate (+). Northern blot hybridization signals were quantified after a 2-day exposure (INH Image Programme). Signals obtained for leaves at time 0 were given the arbitrary value of 1. Values represent the mean of three experiments, and bars indicated the standard errors of the mean. Light/darkness regime affected gene *Hvgrp3* both in (−) and (+) plants.

defense genes: *P. syringae* strain DC3000 induced leaf thionin and pathogenesis-related *HvPr1* genes, while strain 153 had no effect on these genes and pv. *japonica* decreased the expression of *HvLtp4* and thionin genes; *X. campestris* pv. *translucens* and pv. *hordei* both induced gene *HvLtp4*.

Discussion

A number of plant protein families have been identified which have glycine-rich domains and some advances have been made concerning the characterization of the

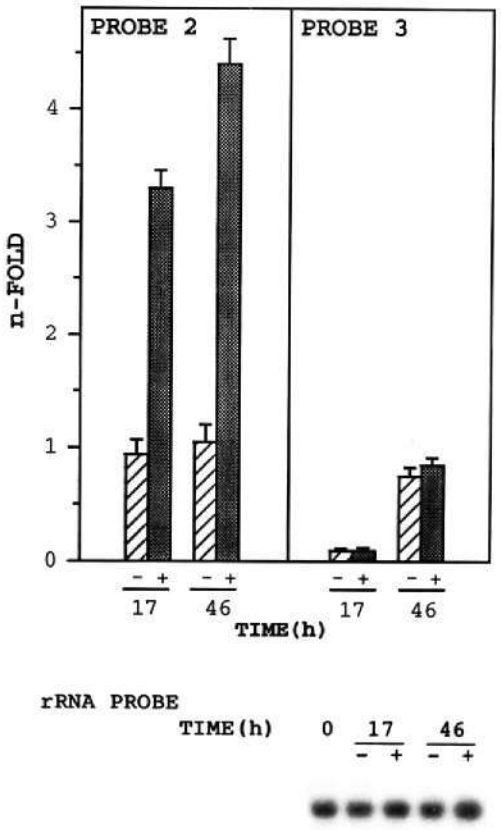

Figure 5. Responses of genes *Hvrp2* and *HvGRP3* to ethylene. Seven-day-old plants were confined in an ethylene-free atmosphere (−) or with 100 ppm ethylene (+) for the times indicated. Northern blot hybridization signals were quantified after 2 days of exposure (INH Image Programme). Signals obtained for leaves at time 0 were given the arbitrary value of 1. Values represent the mean of three experiments, and bars indicated the standard errors of the mean. Light/darkness regime affected gene *Hvgrp3* both in (−) and (+) plants.

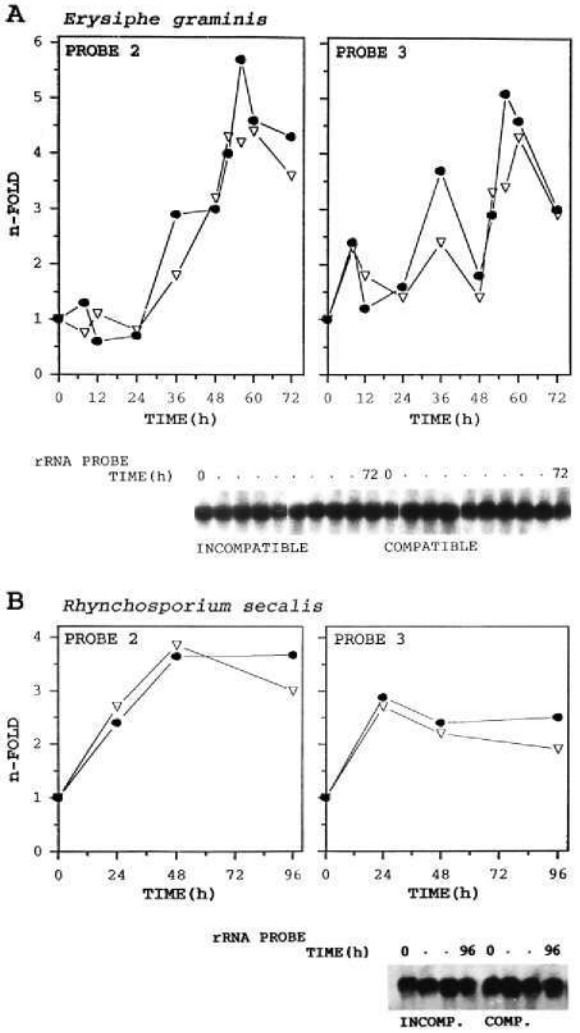

Figure 6. Response of genes *Hvgrp2* and *Hvgrp3* to fungal infection. **A**. Barley cv. Pallas inoculated with incompatible (●) and compatible (▽) strains of *E. graminis*. **B**. Barley cv. Atlas 46 (●; incompatible) and cv. Atlas (▽; compatible) inoculated with *R. secalis*. Northern blot hybridization signals were quantified after a 2-day exposure (INH Image Programma) and represented as *n*-fold increases over basal levels (0 h).

functional roles of some of them. In this context, the expression of the genes under study should be contrasted with that of genes encoding members of the same and of different GRP families. No previous information on the expression of genes encoding CL-GRPs was available, while expression of those corresponding to RNA-GRPs had not been investigated in barley.

The effect of cold on the *Hvgrp3* gene was small – although of the same order as that reported for two other genes encoding RNA-GRPs in *Arabidopsis* [7] – and might have some regulatory significance, as has been postulated for other RNA-GRPs [7, 17], whereas a cell wall reinforcement by an increase of HvGRP2 synthesis could be a plausible response to cold. It is to be noted that neither of the two amino acid sequences includes a cold-shock domain as that found in GRP2s from *A. thaliana* and *N. sylvestris*, which is also present in some bacterial and mammalian transcription factors [21]. Neither drought nor abscisic acid had significant effects on the expression of any of the two *grp* genes, although both positive and negative responses to these stimuli have been reported for different types of GRPs [7, 15, 24, 28, 34]. The lack of a wounding effect on the two *grp* genes was in line with their negative

responses to methyl jasmonate. A negative wounding effect has been reported for an RNA-GRP from tomato [35], while positive responses have been found for other GRPs from this and other families [15, 20, 24, 28, 41].

As already stated, the above experiments were carried out as part of an investigation of the plasticity of stress-related responses in plants. The induction of gene *Hvgrp2* and, to a lesser extent, of gene *Hvgrp3* by fungal pathogens and not by bacterial ones, as well as the variability of their responses to external stimuli, when compared with the responses of the other defense genes investigated (those encoding thionins, LTPs, JIP60, and PRs) point to an involvement of multiple elicitation pathways which interact to affect different gene combinations in different plant-pathogen interactions. A response to viral infection has been previously reported for genes encoding GRPs with a cysteine-rich domain and short glycine-rich domains from petunia [26] and tobacco [46], as well as for a GRP with a cytokeratin-like domain, but lacking the cysteine-rich domain, from rice [13]. A GRP of the latter type from tobacco has been shown to be induced *in vitro* by a fungal glucan elicitor [5]. However, gene *Hvgrp2* did not respond to salycilate treatment, while the GRPs with a cysteine-rich domain from petunia and tobacco do respond [26, 46]. The induction of the *Hvgrp2* gene by ethylene suggests that this hormone could mediate the increased gene expression in response to fungal infection. The suggested cell-wall location of the fungal-responsive CL-GRPS would be in line with the induction by pathogens and mycorrhiza of genes encoding other cell-wall proteins, such as hydroxyproline-rich glycoproteins and proline-rich proteins [3, 19, 39, 40, 43], lipid transfer proteins [14, 30, 45], and defensins [22, 31, 44].

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